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Development and efficacy of novobiocin and rifampicin-resistant *Aeromonas hydrophila* as novel vaccines in channel catfish and Nile tilapia

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ABSTRACT

Three attenuated *Aeromonas hydrophila* vaccines were developed from the virulent 2009 West Alabama isolates through selection for resistance to both novobiocin and rifampicin. When channel catfish (*Ictalurus punctatus*) were IP injected with 4×105 colony-forming unit (CFU) of the mutants, no fish died. However, when the same age and size matched channel catfish were IP injected with similar amount of their virulent parents, 80–100% fish died. Similarly, when Nile tilapia (*Oreochromis niloticus*) were IP injected with 2×108 CFU of the mutants, no fish died. However, when Nile tilapia were IP injected with similar amount of the mutants, all fish died. Vaccination of channel catfish with the mutants at dose of 4×105 CFU/fish offered 86–100% protection against their virulent parents at 14 days post vaccination (dpv). Vaccination of Nile tilapia with the mutants at dose of 2×108 CFU/fish offered 100% protection against their virulent parents at 14, 28, and 56 dpv. Agglutination assay results suggested that protection elicited by the mutants was partially due to antibody-mediated immunity. Taken together, our results suggest that the three attenuated vaccines might be used to protect channel catfish and Nile tilapia against the highly virulent 2009 West Alabama isolates of *A. hydrophila*.

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1. Introduction

Aeromonas hydrophila, a Gram-negative motile bacillus widely distributed in aquatic environments, is a causative agent of motile aeromonad septicaemia (MAS) [1]. MAS is also known as epizootic ulcerative syndrome [2]. The symptoms of *A. hydrophila* infections include swelling of tissues, dropsy, red sores, necrosis, ulceration, and hemorrhagic septicemia [3]. Fish species affected by MAS include tilapia [4,5], catfish [6,7], goldfish [8,9], common carp [10,11], and eel [12]. Although usually considered as a secondary pathogen associated with disease outbreaks, *A. hydrophila* could also become a primary pathogen, causing outbreaks in fish farms with high mortality rates and severe economic losses to the aquaculture industry worldwide [13–18]. In West Alabama, a disease outbreak caused by *A. hydrophila* in 2009 and 2010 has led to an estimated loss of more than $3 million annually [19,20]. Virulence studies have revealed that the 2009 West Alabama isolates of *A. hydrophila* are highly virulent to channel catfish, with LD_{50} values as low as 2×10^2 CFU/fish by intraperitoneal injection [20].

To control disease outbreaks caused by *A. hydrophila*, feeding infected fish with antibiotic-medicated feed is a general practice [21]. However, this practice is expensive and usually ineffective as sick fish tend to remain off feed. In addition, MAS diseases caused by *A. hydrophila* such as the 2009 West Alabama isolates can be very acute, causing mortality within 24 h [19,20]. Furthermore, currently in the US, there are only three FDA approved antibiotics for use in aquaculture: oxytetracycline (Terramycin), sulfadimethoxine (Romet-30), and florfenicol (Aquaflor). The widespread use of the limited number of antibiotics for treating bacterial diseases in aquaculture has led to the development of antibiotic resistance in many fish pathogens worldwide [22]. Therefore, alternative control methods are urgently needed for the aquaculture industry.

Use of vaccine is an alternative control method to prevent MAS. The most extensively studied *A. hydrophila* vaccines are bacterins consisting of formalin or heat-killed bacteria of pathogenic *A. hydrophila* strains [23–25]. In addition, recombinant protein vaccines such as *A. hydrophila* outer membrane proteins and bacterial lysate have been demonstrated to elicit protection against *A. hydrophila* challenges [26–29]. Furthermore, live attenuated vaccines such as *aroA* mutant and transposon Tn916-generated mutant have been reported to confer significant protection against homologous *A. hydrophila* challenge [30,31]. However, it is well known that *A. hydrophila* is very heterogeneous biochemically and serologically, which is the biggest obstacle in developing effective commercial vaccine against *A. hydrophila* [27,32]. To prevent future disease outbreaks caused by the highly virulent West Alabama 2009 isolates of *A. hydrophila*, effective vaccines specific to these isolates are urgently needed.

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Table 1
Aeromonas hydrophila isolates used in this study.

Isolate designation	Year isolated	Location	Species of fish
AL98-C1B	1998	AL, USA	*Ictalurus punctatus*
AL09-71	2009	AL, USA	*Ictalurus punctatus*
AL09-72	2009	AL, USA	*Ictalurus punctatus*
AL09-73	2009	AL, USA	*Ictalurus punctatus*

To develop effective live attenuated bacterial vaccines, rifampicin-resistant strategy has been successfully used to develop the two commercially available vaccines in aquaculture: *Edwardsiella ictaluri* (AquaVac-ESC) and *Flavobacterium columnare* (AquaVac-COL) [33,34]. However, it is not clear whether the rifampicin-resistant strategy or other antibiotic-resistant strategy could also be used to attenuate *A. hydrophila* for the purpose of novel vaccine development. Novobiocin, a natural antibiotic produced by the actinomycete *Streptomyces niveus*, is a member of the order *Actinobacteria* [35]. Novobiocin works as a natural inhibitor of bacterial DNA gyrase, resulting in bacterial cell-death [36]. DNA gyrase, an ATP-dependent enzyme that acts by creating a transient double-stranded DNA break, is essential for efficient DNA replication, transcription, and recombination by catalyzing the negative supercoiling of DNA [37]. Recently, novobiocin-resistance strategy has been successfully used to attenuate Gram-positive *Streptococcus iniae* and Gram-negative *E. ictaluri* [38,39]. Therefore, the objectives of this study were to determine: (1) whether resistance to rifampicin or to novobiocin or to a combination of the two antibiotics (hereafter called N + R) could be used to attenuate virulent *A. hydrophila*; and (2) whether attenuated *A. hydrophila* could be used as live vaccine to protect channel catfish or Nile tilapia from infections by its virulent parent *A. hydrophila*.

2. Materials and methods

2.1. Induction of antibiotic resistance in A. hydrophila

A. hydrophila AL98-C1B isolate obtained from MAS diseased channel catfish was used to determine whether resistance to rifampicin or to novobiocin or to N + R was able to attenuate *A. hydrophila* AL98-C1B. After the most effective attenuation strategy was determined to be N + R, the N + R strategy was then used to attenuate the three highly virulent 2009 isolates (Table 1) of *A. hydrophila*. The identification of the four isolates has been published previously [19]. The archived isolates were recovered from frozen stocks (2 mL aliquots stored at −80 °C) and grown in tryptic soy broth (TSB) (Fisher Scientific, Pittsburgh, PA) for 24 h at 28 °C. Novobiocin sodium salt and rifampicin were purchased from Promega (Madison, WI) and Sigma–Aldrich (St. Louis, MO), respectively. All *A. hydrophila* isolates were cultured in tryptic soy broth (TSB) containing different concentrations of N + R for 24 h at 28 °C. The initial concentration of N + R that allowed growth of *A. hydrophila* was 12.5 μg/mL. After 20 passages of *A. hydrophila* in TSB containing the same or higher concentration of N + R, all three strains of *A. hydrophila* were able to grow in TSB containing 1600 μg/mL of N + R. The three parent and N + R-resistant *A. hydrophila* strains were then grown on 5% sheep blood agar plates (Thermo Fisher Scientific Remel Products, Lenexa, KS) for bacterial identification. Bacteria isolates were identified by API 20E test (BioMerieux, Durham, NC) and gas chromatography analysis of fatty acid methyl ester using MIDI microbial identification system (MIDI, Newark, DE) according to established procedures [40].

2.2. Virulence of antibiotic-resistant A. hydrophila isolate to channel catfish

To study the virulence of antibiotic-resistant *A. hydrophila* isolates to channel catfish compared to their parent isolates, all bacteria were cultured overnight in TSB at 28 °C. An optical density (OD) of 1.0 of the bacterial cultures was measured at 540 nm using Thermospectronic spectrophotometer (Fisher Scientific, Pittsburgh, PA). Serial dilutions of each isolate (in triplicates) were then immediately prepared in TSB and 100 μL of serially diluted *A. hydrophila* was plated onto TSA plates immediately. After the TSA plates were incubated at 28 °C for 24 h, the average number of colony forming unit (CFU) per milliliter was then calculated for all isolates. Five different doses (1.8×10^8, 1.2×10^8, 2.4×10^7, 1.2×10^7, and 6×10^6 CFU/fish) were chosen to determine whether AL98-C1B mutants were attenuated based on the LD_{50} value of the parent *A. hydrophila* AL98-C1B to channel catfish (4.6 ± 1.3 g) published previously (LD_{50} of AL98-C1B = 3.6×10^6 CFU/fish) [20]. Four different doses (4×10^5, 2×10^5, 1×10^5, and 5×10^4 CFU/fish) were used to determine whether any antibiotic resistant 2009 isolates of *A. hydrophila* were attenuated based on LD_{50} value of the 2009 isolates to channel catfish (4.6 ± 1.3 g) published previously (LD_{50} ranged from 2.0×10^2 to 1.6×10^3 CFU/fish) [20]. Channel catfish (mean weight of 7.0 ± 1.6 g) were exposed to parent or antibiotic-resistant *A. hydrophila* AL98-C1B through intraperitoneal (IP) injection. All channel catfish (industry pool strain, USDA, ARS, Catfish Genetics Research Unit, Stoneville, MS) used in this study were raised at the USDA ARS Aquatic Animal Health Research facility located at Auburn, AL. A total of 60 fish were used in each treatment group (20 fish per tank, three replicates). All fish treatment protocols were approved by Institutional Animal Care and Use Committee of the Aquatic Animal Health Research Laboratory following related guidelines. Fish were acclimated in flow-through 57-L aquaria supplied with 0.5 L h^{-1} dechlorinated water for 10 days prior to experiments. A 12:12 h light:dark period was maintained, and supplemental aeration was supplied by an air stone. Fish were fed 3% body weight daily with commercial dry fish food. During the experiment, the mean dissolved oxygen was 5.6 mg L^{-1}, temperature was 26 °C, pH was 7.1 and hardness was 100 mg L^{-1}. Mortalities were recorded daily for 14 days post exposure to *A. hydrophila* and the presence or absence of *A. hydrophila* in dead fish was determined from bacterial culture derived from the brain and kidney samples on blood agar plates followed by API-20E tests (Biomérieux, Durham, NC).

2.3. Virulence of N + R-resistant 2009 isolate of A. hydrophila to Nile tilapia

To study the virulence of N + R-resistant 2009 isolates of *A. hydrophila* to Nile tilapia compared to their parent isolates, all bacteria were cultured overnight in TSB at 28 °C. An optical density (OD) of 1.0 of the bacterial cultures was measured at 540 nm and the average number of CFU/mL for each culture was then calculated. Three different doses (2×10^8, 2×10^7, and 2×10^6 CFU/fish) were used to determine whether antibiotic-resistant mutants were attenuated. Parent and antibiotic-resistant 2009 isolates of *A. hydrophila* were exposed to Nile tilapia (*Oreochromis niloticus*, mean weight of 10.4 ± 0.6 g) through intraperitoneal (IP) injection. All Nile tilapia (Aquasafra, Bradenton, FL) used in this study were raised at the USDA ARS Aquatic Animal Health Research facility located at Auburn, AL. A total of 60 fish were used in each treatment group (20 fish per tank, three replicates). Mortalities were recorded daily for 14 days post exposure to *A. hydrophila* and the presence or absence of *A. hydrophila* in dead fish was determined from bacterial culture

derived from the brain and kidney samples on blood agar plates followed by API-20E tests.

2.4. Vaccination of fish with N + R-resistant 2009 isolates of A. hydrophila followed by challenge with virulent parents

Attenuated N + R-resistant *A. hydrophila* vaccines were cultured in TSB broth at 28 °C with shaking at 125 rpm overnight before vaccination. Channel catfish were vaccinated with four different doses (4×10^5, 2×10^5, 1×10^5, and 5×10^4 CFU/fish) of antibiotic-resistant *A. hydrophila* in a total volume of 100 μL by IP injection. The four vaccination doses were chosen because the vaccines at those doses failed to kill any channel catfish based on the results from the virulence studies. Nile tilapia fish were vaccinated with three different doses (2×10^8, 2×10^7, and 2×10^6 CFU/fish) of N + R-resistant mutants in a total volume of 100 μL by IP injection. As sham-vaccination controls, 100 μL of TSB was injected into each fish. A total of 60 fish were used in each treatment group (20 fish per tank, three replicates). At 14, 28, and 56 days post vaccination (dpv), fish were challenged with the parent isolate of *A. hydrophila* through IP injection. Mortalities were recorded for 14 days post challenge and the presence or absence of *A. hydrophila* in dead fish was determined as described earlier. Results of challenge were presented as relative percent of survival (RPS) as described previously [41]. RPS was calculated according to the following formula: RPS = [1 − (vaccinated mortality/control mortality)] × 100.

2.5. Minimum effective vaccination dose of N + R-resistant mutants in channel catfish or Nile tilapia

To determine the minimum effective dose of N + R-resistant 2009 isolates of *A. hydrophila* in channel catfish by IP vaccination, five doses (2×10^5, 2×10^4, 2×10^3, 2×10^2, and 2×10^1 CFU/fish) in a total volume of 100 μL were IP injected to channel catfish. A total of 60 fish were used in each treatment group (20 fish per tank, three replicates). To determine the minimum vaccination dose to channel catfish by immersion, three concentrations (2×10^7, 2×10^6, and 2×10^5 CFU/mL) in a total volume of 5 L were used to immerse channel catfish for 2 h in the presence of 0.02% Tween-20. As sham-vaccination control, fish were injected or bath immersed with tryptic soy broth. At 21 dpv, fish were challenged with virulent parent isolate through IP injection. To determine the minimum effective dose of N + R-resistant 2009 isolates of *A. hydrophila* in Nile tilapia by IP vaccination, six doses (2×10^8, 2×10^7, 2×10^6, 2×10^5, 2×10^4, and 2×10^3 CFU/fish) in a total volume of 100 μL were IP injected to each fish. A total of 60 fish were used in each treatment group (20 fish per tank, three replicates). To determine the minimum vaccination dose to Nile tilapia by immersion, three concentrations (2×10^7, 2×10^6, and 2×10^5 CFU/mL) in a total volume of 5 L were used to immerse channel catfish for 2 h in the presence of 0.02% Tween-20. As sham-vaccination control, fish were injected or bath immersed with tryptic soy broth. At 28 dpv, fish were challenged with virulent parent isolate through IP injection. Mortalities were recorded for 14 days post challenge and the presence or absence of *A. hydrophila* in dead fish was determined as described earlier. Results of challenge were presented as RPS.

2.6. Antibody titration by bacterial agglutination assay

At 14 or 28 days post vaccination, a total of 15 fish from each vaccination group (TSB- or antibiotic resistant *A. hydrophila*-vaccinated) were bled to obtain serum. Agglutination assay was performed using published procedures [42] with slight modifications. Briefly, the 2009 isolates of *A. hydrophila* (AL09-71, AL09-72, and AL09-73) were grown in TSB overnight at 28 °C with shaking at 125 rpm. The bacteria were harvested by centrifugation at 3000 rpm for 15 min and the bacterial pellets were washed twice with PBS (pH 7.2) followed by centrifugation. PBS-washed bacterial pellets were resuspended in PBS again and heat inactivated at 60 °C for 1 h. The heat inactivated bacteria were then washed twice with PBS followed by centrifugation and resuspended in PBS. The optical density of heat-inactivated bacteria in PBS was then adjusted to 0.4 at 540 nm. Serum from *A. hydrophila* vaccinated fish or TSB-sham vaccinated fish was collected at 14 and 28 dpv. To serially dilute the serum, 25 μL of PBS was added to all wells, and 25 μL of 1:1 PBS diluted serum was added to the first well. A twofold serial dilution of serum was then performed by taking 25 μL solution from the first well and added to the second well and so on until the last well. A volume of 25 μL was then taken from the last well and discarded so that each well contained a final volume of 25 μL. After serial dilution of serum was completed, 25 μL of heat-inactivated *A. hydrophila* in PBS was then added to each well. The microtiter plate was incubated at 37 °C overnight. The highest dilution of serum sample that showed positive agglutination was recorded and expressed as $\log_2$ antibody titer of the serum. Serum from *A. hydrophila* infected fish at 28 day post infection was used as positive control and PBS was used as negative controls.

Table 2
Virulence of parent and antibiotic-resistant mutants of *A. hydrophila* AL98-C1B to channel catfish by intraperitoneal injection.

Isolate name	Injection dose (CFU/fish)	Mortality (%)
AL98-C1B Parent	1.8×10^8	97.5
AL98-C1B Parent	1.2×10^8	97.5
AL98-C1B Parent	2.4×10^7	75
AL98-C1B Parent	1.2×10^7	65
AL98-C1B Parent	6.0×10^6	52.5
AL98-C1B Novo[a]	1.8×10^8	97.5
AL98-C1B Novo	1.2×10^8	95
AL98-C1B Novo	2.4×10^7	92.5
AL98-C1B Novo	1.2×10^7	12.5
AL98-C1B Novo	6.0×10^6	12.5
AL98-C1B Rifa[b]	1.8×10^8	17.5
AL98-C1B Rifa	1.2×10^8	0
AL98-C1B Rifa	2.4×10^7	0
AL98-C1B Rifa	1.2×10^7	0
AL98-C1B Rifa	6.0×10^6	0
AL98-C1B N + R[c]	1.8×10^8	0
AL98-C1B N + R	1.2×10^8	0
AL98-C1B N + R	2.4×10^7	0
AL98-C1B N + R	1.2×10^7	0
AL98-C1B N + R	6.0×10^6	0

[a] Novo, novobiocin resistant isolate.
[b] Rifa, rifampicin resistant isolate.
[c] N + R, novobiocin and rifampicin resistant isolate.

2.7. Statistical analysis

All statistical analyses were performed using SigmaStat 3.5 software (Systat Software, Inc., Point Richmond, CA). Differences in antibody titer and mortality were analyzed with Student's *t*-test and the significance level was defined as $P < 0.05$.

3. Results

3.1. Attenuation of AL98-C1B using different antibiotic-resistant strategies

Results on the attenuation of AL98-C1B using three different antibiotic-resistant strategies are summarized in Table 2. All mortality data had standard deviation <10%, therefore, mean mortality was used throughout this study. All dead fish after exposure

Table 3
Cumulative mortality and relative percent survival of AL98-C1B N + R vaccinated channel catfish challenged with virulent isolates of *A. hydrophila*.

Vaccination[a]	Vaccine dose (CFU/fish)	Isolate used for challenge	Challenge dose (CFU/fish)	dpv[b]	Mortality (%)	RPS[c] (%)
Trial I						
Sham TSB	–	AL98-C1B	1.0×10^7	37	60	–
AL98-C1B N + R[d]	1.2×10^8	AL98-C1B	1.0×10^7	37	65	0
Sham TSB	–	AL09-71	1.2×10^5	37	100	–
AL98-C1B N + R	1.2×10^8	AL09-71	1.2×10^5	37	50	50
Sham TSB	–	AL09-72	3.4×10^5	37	80	–
AL98-C1B N + R	1.2×10^8	AL09-72	3.4×10^5	37	80	0
Sham TSB	–	AL09-73	4.4×10^5	37	60	–
AL98-C1B N + R	1.2×10^8	AL09-73	4.4×10^5	37	70	0
Trial II						
Sham TSB	–	AL98-C1B	1.2×10^7	38	70	–
AL98-C1B N + R	1.4×10^8	AL98-C1B	1.0×10^7	38	70	0
Sham TSB	–	AL09-71	1.0×10^5	38	90	–
AL98-C1B N + R	1.4×10^8	AL09-71	1.3×10^5	38	60	33
Sham TSB	–	AL09-72	3.3×10^5	38	70	–
AL98-C1B N + R	1.4×10^8	AL09-72	3.3×10^5	38	80	0
Sham TSB	–	AL09-73	4.6×10^5	38	80	–
AL98-C1B N + R	1.4×10^8	AL09-73	4.6×10^5	38	80	0

[a] Vaccination through intraperitoneal injection.
[b] dpv, days post vaccination.
[c] RPS, relative percent of survival.
[d] N + R, novobiocin and rifampicin resistant isolate.

to parents or mutants of *A. hydrophila* throughout this study were culture positive for *A. hydrophila*. At the highest injection dose (1.8×10^8 CFU/fish), the parent and the novobiocin-resistant mutant both killed 97.5% fish, whereas the rifampicin-resistant mutant killed 17.5% fish. However, when N + R-resistant mutant was injected to fish at all doses, no fish died (Table 2). At injection dose of 1.2×10^8 CFU/fish, the parent and the novobiocin-resistant mutant killed 97.5% and 95% fish, respectively. However, when similar amount of rifampicin- or N + R-resistant mutant was injected to the same size and age matched channel catfish, no fish died. At the two lower injection doses (1.2×10^7 and 6.0×10^6 CFU/fish), novobiocin-resistant mutant killed 12.5% fish, whereas the parent killed 65% and 52.5% fish, respectively (Table 2). Of the three attenuation methods, N + R-resistant strategy appeared to be the best. The second best attenuation method was the rifampicin-resistant strategy. Of the three attenuation methods, novobiocin-resistant strategy had the least effect on attenuation of AL98-C1B.

Table 4
Virulence of parents and mutants of the three 2009 West Alabama isolates of *A. hydrophila* to channel catfish by intraperitoneal injection.

Isolate name	Injection dose (CFU/catfish)	Mortality (%)
AL09-71 Parent	4×10^5	80
AL09-71 N + R[a]	4×10^5	0
AL09-72 Parent	4×10^5	100
AL09-72 N + R	4×10^5	0
AL09-73 Parent	4×10^5	100
AL09-73 N + R	4×10^5	0
AL09-71 Parent	2×10^5	80
AL09-71 N + R	2×10^5	0
AL09-72 Parent	2×10^5	90
AL09-72 N + R	2×10^5	0
AL09-73 Parent	2×10^5	90
AL09-73 N + R	2×10^5	0
AL09-71 Parent	1×10^5	80
AL09-71 N + R	1×10^5	0
AL09-72 Parent	1×10^5	80
AL09-72 N + R	1×10^5	0
AL09-73 Parent	1×10^5	80
AL09-73 N + R	1×10^5	0
AL09-71 Parent	5×10^4	70
AL09-71 N + R	5×10^4	0
AL09-72 Parent	5×10^4	70
AL09-72 N + R	5×10^4	0
AL09-73 Parent	5×10^4	70
AL09-73 N + R	5×10^4	0

[a] N + R, novobiocin and rifampicin resistant isolate.

3.2. Vaccination of channel catfish with N + R-resistant AL98-C1B followed by challenge with virulent parent

The N + R-resistant AL98-C1B that was safe to channel catfish at all injected doses tested above was then evaluated as vaccine. When N + R-resistant AL98-C1B vaccinated channel fish were challenged with their virulent parent isolate at 37 or 38 dpv, relative percent of survival of vaccinated fish was 0% (Table 3). Similarly, when N + R-resistant AL98-C1B vaccinated fish were challenged with *A. hydrophila* AL09-72 or AL09-73 at 37 or 38 dpv, RPS values were 0% (Table 3). However, when N + R-resistant AL98-C1B vaccinated fish were challenged with *A. hydrophila* AL09-71 at 37 and 38 dpv, RPS values were 50% and 33%, respectively (Table 3).

3.3. Attenuation of 2009 isolates of A. hydrophila using N + R-resistance strategy and their virulence to channel catfish or Nile tilapia

N + R resistance strategy was chosen to attenuate the 2009 isolates due to the fact that it was the most effective method to attenuate *A. hydrophila* AL98-C1B. Attenuated 2009 isolates of *A. hydrophila* shared the same biochemical profile as their virulent parent s by API 20E test, which was β-galactosidase positive, arginine dihydrolase positive, lysine decarboxylase negative, ornithine decarboxylase negative, citrate utilization positive, H_2S production negative, urease negative, tryptophan deaminase negative, indole production positive, acetoin production positive, gelatinase positive, glucose oxidation positive, mannitol oxidation positive, inositol oxidation positive, sorbitol oxidation negative, rhamnose oxidation negative, saccharose oxidation positive, melibiose oxidation negative, amygdalin oxidation negative, arabinose

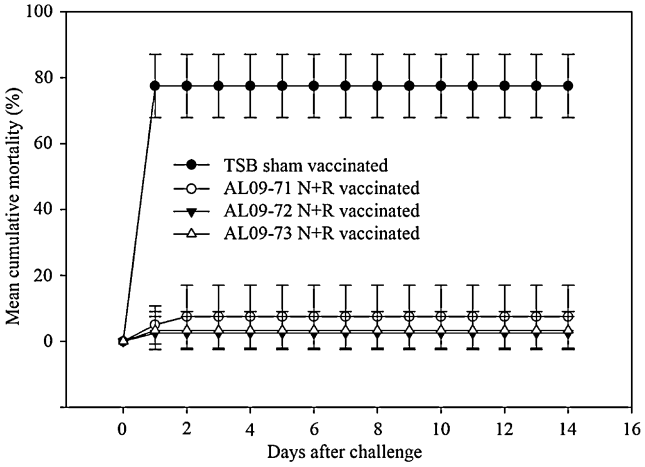

Fig. 1. Daily mean percent cumulative mortality of channel catfish intraperitoneally vaccinated with or without the novobiocin and rifampicin resistant mutants of *Aeromonas hydrophila* and challenged with their respective virulent parent isolates of *A. hydrophila* through intraperitoneal injection at 14 days post vaccination. Daily mean percent cumulative mortalities were calculated from vaccine trials at four vaccination doses (4.0×10^5, 2.0×10^5, 1.0×10^5, and 5.0×10^4). Data are presented as mean ± S.D. from the four trials.

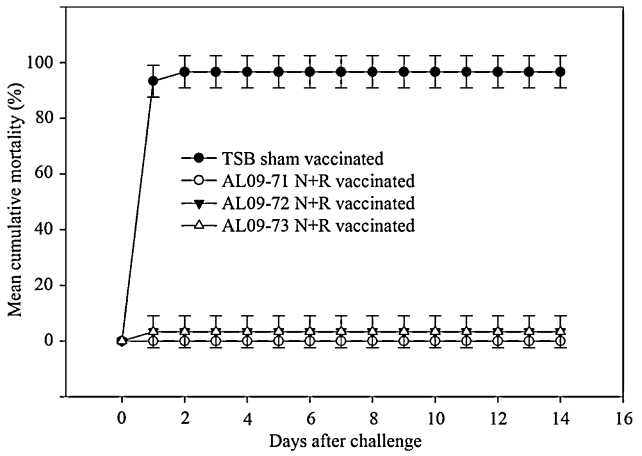

Fig. 2. Daily mean percent cumulative mortality of Nile tilapia intraperitoneally vaccinated with or without the novobiocin and rifampicin resistant mutants of *Aeromonas hydrophila* and challenged with their respective virulent parent isolates of *A. hydrophila* through intraperitoneal injection at 14 days post vaccination. Daily mean percent cumulative mortalities were calculated from vaccine trials at three vaccination doses (2.0×10^8, 2.0×10^7, and 2.0×10^6). Data are presented as mean ± S.D. from the three trials.

oxidation positive, and cytochrome oxidase positive. However, the N + R-resistant mutants grew much slower than their respective parents when similar amounts of both were streaked onto 5% blood agar plates or plates onto tryptic soy agar plates. Results on the virulence of the N + R resistant mutants compared to their virulent parents are summarized in Table 4. At injection dose of 4×10^5 CFU/fish, the three virulent parents killed 80–100% fish. However, at the same injection dose, the N + R-resistant mutants of *A. hydrophila* caused no mortality to channel catfish (Table 4). Similarly, at injection doses of 2×10^5, 1×10^5, and 5×10^4 CFU/fish, the virulent parent isolates killed 70–90% fish, whereas the N + R-resistant mutants killed no fish (Table 4). Virulence of N + R-resistant 2009 isolates of *A. hydrophila* to Nile tilapia is summarized in Table 5. At injection dose of 2×10^8 CFU/fish, the three virulent parents all killed 100% fish. However, at the same injection dose, the N + R-resistant mutants of *A. hydrophila* caused no mortality to Nile tilapia (Table 5). Similarly, at injection doses of 2×10^7 CFU/fish, the virulent parents killed 90–100% fish, whereas the N + R-resistant mutants killed no fish (Table 5). At injection dose of 2×10^6 CFU/fish, both parents and mutants were avirulent to Nile tilapia.

Table 5
Virulence of parents and mutants of the three 2009 West Alabama isolates of *A. hydrophila* to Nile tilapia by intraperitoneal injection.

Isolate name	Injection dose (CFU/tilapia)	Mortality (%)
AL09-71 Parent	2×10^8	100
AL09-71 N + R[a]	2×10^8	0
AL09-72 Parent	2×10^8	100
AL09-72 N + R	2×10^8	0
AL09-73 Parent	2×10^8	100
AL09-73 N + R	2×10^8	0
AL09-71 Parent	2×10^7	100
AL09-71 N + R	2×10^7	0
AL09-72 Parent	2×10^7	90
AL09-72 N + R	2×10^7	0
AL09-73 Parent	2×10^7	90
AL09-73 N + R	2×10^7	0
AL09-71 Parent	2×10^6	0
AL09-71 N + R	2×10^6	0
AL09-72 Parent	2×10^6	0
AL09-72 N + R	2×10^6	0
AL09-73 Parent	2×10^6	0
AL09-73 N + R	2×10^6	0

[a] N + R, novobiocin and rifampicin resistant isolate.

3.4. Vaccination with N + R-resistant 2009 isolates of A. hydrophila followed by challenge with virulent parents

When N + R-resistant AL09-71 vaccinated channel fish were challenged with their virulent parent at 14 dpv, relative percent of survival of vaccinated fish at the four vaccination doses ranged from 71% to 100% (Table 6). Similarly, when N + R-resistant AL09-73 vaccinated fish were challenged with *A. hydrophila* AL09-73 at 14 dpv, RPS values of vaccinated fish at the four vaccination doses were 86–100% (Table 6). When N + R-resistant AL09-72 vaccinated channel fish were challenged with their virulent parent at 14 dpv, RPS values were all 100% (Table 6). At 14dpv, when all channel catfish were challenged with virulent parents, cumulative mortalities of N + R mutants vaccinated fish at different time points were significantly ($P < 0.05$) lower than those of TSB sham-vaccinated fish (Fig. 1), suggesting that these mutants provided significant protection against their respective parent. At vaccination dose of 5×10^4 CFU/fish, when vaccinated fish were challenged with their respective virulent parent at 28 and 56 dpv, RPS values of vaccinated fish ranged from 43% to 75% and from 42% to 60%, respectively (Table 6).

When N + R-resistant AL09-71 vaccinated Nile tilapia were challenged with their virulent parent at 14 dpv, RPS values of vaccinated fish at the three vaccination doses were all 100% (Table 7). When N + R-resistant AL09-72 or AL09-73 vaccinated fish were challenged with their virulent parent strain of *A. hydrophila* at 14 dpv, RPS values ranged from 89% to 100% (Table 7). At 14 dpv, when all Nile tilapia were challenged with virulent parents, cumulative mortalities of N + R mutants vaccinated fish at different time points were significantly ($P < 0.05$) lower than those of TSB sham-vaccinated fish (Fig. 2), suggesting that these mutants provided significant protection to Nile tilapia against their respective parents. When vaccinated fish were challenged with virulent parent at 28 and 56 dpv, RPS values were all 100% at all three vaccination doses tested (Table 7).

Table 6
Cumulative mortality and relative percent survival of the N + R mutants vaccinated channel catfish challenged with virulent parent isolates of *A. hydrophila*.

Vaccination[a]	Vaccine dose (CFU/fish)	Challenge isolate	Challenge dose (CFU/fish)	dpv[b]	Mortality (%)	RPS[c] (%)
Sham TSB	–	AL09-71	5.0×10^4	14	70	–
AL09-71 N + R[d]	4.0×10^5	AL09-71	5.0×10^4	14	10	86
AL09-71 N + R	2.0×10^5	AL09-71	5.0×10^4	14	0	100
AL09-71 N + R	1.0×10^5	AL09-71	5.0×10^4	14	20	71
AL09-71 N + R	5.0×10^4	AL09-71	5.0×10^4	14	0	100
Sham TSB	–	AL09-72	5.0×10^4	14	70	–
AL09-72 N + R	4.0×10^5	AL09-72	5.0×10^4	14	0	100
AL09-72 N + R	2.0×10^5	AL09-72	5.0×10^4	14	0	100
AL09-72 N + R	1.0×10^5	AL09-72	5.0×10^4	14	0	100
AL09-72 N + R	5.0×10^4	AL09-72	5.0×10^4	14	0	100
Sham TSB	–	AL09-73	5.0×10^4	14	70	–
AL09-73 N + R	4.0×10^5	AL09-73	5.0×10^4	14	0	100
AL09-73 N + R	2.0×10^5	AL09-73	5.0×10^4	14	0	100
AL09-73 N + R	1.0×10^5	AL09-73	5.0×10^4	14	10	86
AL09-73 N + R	5.0×10^4	AL09-73	5.0×10^4	14	0	100
Sham TSB	–	AL09-71	5.0×10^4	28	80	–
AL09-71 N + R[d]	5.0×10^4	AL09-71	5.0×10^4	28	20	75
Sham TSB	–	AL09-72	5.0×10^4	28	75	–
AL09-72 N + R	5.0×10^4	AL09-72	5.0×10^4	28	20	73
Sham TSB	–	AL09-73	5.0×10^4	28	70	–
AL09-73 N + R	5.0×10^4	AL09-73	5.0×10^4	28	40	43

[a] Vaccination through intraperitoneal injection.
[b] dpv, days post vaccination.
[c] RPS, relative percent of survival.
[d] N + R, novobiocin and rifampicin resistant isolate.

3.5. Minimum effective vaccination dose of AL09-71 N + R in catfish or tilapia

Results of experiments to determine minimum vaccination dose of AL09-71 N + R mutant to protect channel catfish from infection by its virulent parent are summarized in Table 8. When IP vaccinated fish were challenged with their virulent parent at 21 dpv, RPS values were 100% when vaccination doses were 2×10^4 and 2×10^5 CFU/fish (Table 8). When channel catfish were vaccinated at doses of 2×10^3 or 2×10^2 CFU/fish by IP, RPS values were 89% and 78%, respectively. At the lowest vaccination dose (2×10^1 CFU/fish) tested, RPS value dropped to 44%. When channel catfish were vaccinated by AL09-71 N + R through bath immersion, RPS of vaccinated fish at 21 dpv ranged from 50% to 75% (Table 8).

Results of experiments to determine minimum vaccination dose of AL09-71 N + R mutant to protect Nile tilapia from infection by its virulent parent are summarized in Table 9. When IP vaccinated fish were challenged with their virulent parent at 28 dpv, RPS values

Table 7
Cumulative mortality and relative percent survival of the N + R mutants vaccinated Nile tilapia challenged with virulent parent isolates of *A. hydrophila*.

Vaccination[a]	Vaccine dose (CFU/fish)	Isolate used for challenge	Challenge Dose (CFU/fish)	dpv[b]	Mortality (%)	RPS[c] (%)
Sham TSB	–	AL09-71	2.0×10^7	14	100	–
AL09-71 N + R[d]	2.0×10^8	AL09-71	2.0×10^7	14	0	100
AL09-71 N + R	2.0×10^7	AL09-71	2.0×10^7	14	0	100
AL09-71 N + R	2.0×10^6	AL09-71	2.0×10^7	14	0	100
Sham TSB	–	AL09-72	2.0×10^7	14	90	–
AL09-72 N + R	2.0×10^8	AL09-72	2.0×10^7	14	0	100
AL09-72 N + R	2.0×10^7	AL09-72	2.0×10^7	14	10	89
AL09-72 N + R	2.0×10^6	AL09-72	2.0×10^7	14	0	100
Sham TSB	–	AL09-73	2.0×10^7	14	90	–
AL09-73 N + R	2.0×10^8	AL09-73	2.0×10^7	14	0	100
AL09-73 N + R	2.0×10^7	AL09-73	2.0×10^7	14	10	89
AL09-73 N + R	2.0×10^6	AL09-73	2.0×10^7	14	0	100
Sham TSB	–	AL09-71	2.0×10^7	28	100	–
AL09-71 N + R[d]	2.0×10^8	AL09-71	2.0×10^7	28	0	100
AL09-71 N + R	2.0×10^7	AL09-71	2.0×10^7	28	0	100
AL09-71 N + R	2.0×10^6	AL09-71	2.0×10^7	28	0	100
Sham TSB	–	AL09-72	2.0×10^7	28	90	–
AL09-72 N + R	2.0×10^8	AL09-72	2.0×10^7	28	0	100
AL09-72 N + R	2.0×10^7	AL09-72	2.0×10^7	28	0	100
AL09-72 N + R	2.0×10^6	AL09-72	2.0×10^7	28	0	100
Sham TSB	–	AL09-73	2.0×10^7	28	90	–
AL09-73 N + R	2.0×10^8	AL09-73	2.0×10^7	28	0	100
AL09-73 N + R	2.0×10^7	AL09-73	2.0×10^7	28	0	100
AL09-73 N + R	2.0×10^6	AL09-73	2.0×10^7	28	0	100

[a] Vaccination through intraperitoneal injection.
[b] dpv, days post vaccination.
[c] RPS, relative percent of survival.
[d] N + R, novobiocin and rifampicin resistant isolate.

Table 8
Minimum effective vaccination dose of AL09-71 N + R to protect channel catfish from challenges by virulent parent *A. hydrophila* AL09-71.

Vaccine group	Vaccination route	Vaccination dose[a]	Challenge Dose (CFU/fish)	dpv[b]	Mortality (%)	RPS[e] (%)
Sham TSB	IP[c]	–	2.0×10^5	21	90	–
AL09-71 N + R	IP	2.0×10^5	2.0×10^5	21	0	100
AL09-71 N + R	IP	2.0×10^4	2.0×10^5	21	0	100
AL09-71 N + R	IP	2.0×10^3	2.0×10^5	21	10	89
AL09-71 N + R	IP	2.0×10^2	2.0×10^5	21	20	78
AL09-71 N + R	IP	2.0×10^1	2.0×10^5	21	50	44
Sham TSB	IM[d]	–	2.0×10^5	21	80	–
AL09-71 N + R	IM	2.0×10^7	2.0×10^5	21	30	63
AL09-71 N + R	IM	2.0×10^6	2.0×10^5	21	20	75
AL09-71 N + R	IM	2.0×10^5	2.0×10^5	21	20	75
AL09-71 N + R	IM	2.0×10^4	2.0×10^5	21	40	50

[a] Vaccination dose for IP and IM was in the unit of CFU/fish and CFU/mL, respectively.
[b] Days post vaccination.
[c] Intraperitoneal injection.
[d] Bath immersion.
[e] Relative percent of survival.

were 100% when vaccination doses were 2×10^6 CFU/fish or higher (Table 9). When Nile tilapia were IP vaccinated with AL09-71 N + R at doses of 2×10^5 CFU/fish or lower, RPS values dropped significantly ($P < 0.05$) to 8–25%. When Nile tilapia were vaccinated by AL09-71 N + R through bath immersion, RPS of vaccinated fish at 28 dpv ranged from 4% to 38% (Table 9).

3.6. Agglutination assay results

Agglutination assay results are summarized in Fig. 3. At 14 and 28 dpv, antibody titers of N + R mutant vaccinated channel fish appeared to have higher antibody titers than those of sham TSB vaccinated fish (Fig. 3A). However, there was no significant difference between N + R mutant of AL09-71 or AL09-72 and the TSB sham vaccination control at either 14 dpv or 28 dpv (Fig. 3A). The only significant difference observed was between the TSB sham vaccination control and the N + R mutant of AL09-73 vaccinated fish at 14 dpv (Fig. 3A). Similarly, at 14 and 28 dpv, antibody titers of N + R mutant vaccinated Nile tilapia appeared to have higher antibody titers than those of sham TSB vaccinated fish on average (Fig. 3B). At 14 dpv, all N + R mutant vaccinated fish had significantly higher antibody titer than that of control fish except the N + R AL09-72 mutant vaccinated fish (Fig. 3B). At 28 dpv, all N + R mutant vaccinated fish had significantly higher antibody titer than that of control fish except the N + R AL09-71 mutant vaccinated fish (Fig. 3B).

4. Discussion

Using novobiocin and rifampicin-resistant strategy, three N + R resistant *A. hydrophila* mutants were obtained from the 2009 West Alabama virulent isolates. The virulent parents were only able to grow in TSB containing 12.5 μg/μL of novobiocin and rifampicin, whereas the mutants were able to grow in TSB containing 1600 μg/μL of novobiocin and rifampicin, suggesting that the mutants were at least 128 times more resistant to novobiocin and rifampicin than their parents. Virulence studies revealed that the mutants were avirulent to channel catfish at dose of 4×10^5 CFU/fish, whereas the LD_{50} of the parents ranged from 2.0×10^2 to 1.6×10^3 CFU/fish [20], suggesting that the mutants were attenuated at least 250-fold. The attenuation of virulence of the mutants could be due to their fitness cost resulting from their resistance to N + R. Decreased virulence as a fitness cost has been reported in *Staphylococcus aureus* associated with antibiotic resistance [43]. Differential transcriptome analysis on teicoplanin-resistant *S. aureus* has revealed that as resistance to antibiotic teicoplanin increased, some virulence-associated genes are down-regulated [43]. Growth studies revealed that N + R mutants had much lower growth rate as revealed by their colony sizes on agar plates compared to that of their parents, suggesting that their slow growth rate is a fitness cost resulting from resistance to N + R. Slower growth is well known as a fitness cost in antibiotic resistant bacteria [44]. For example, the macrolide-resistant *Campylobacter*

Table 9
Minimum effective vaccination dose of AL09-71 N + R to protect Nile tilapia from challenges by virulent parent *A. hydrophila* AL09-71.

Vaccine group	Vaccination route	Vaccination dose[a]	Challenge Dose (CFU/fish)	dpv[b]	Mortality (%)	RPS[e] (%)
Sham TSB	IP[c]	–	2.0×10^7	28	100	–
AL09-71 N + R	IP	2.0×10^8	2.0×10^7	28	0	100
AL09-71 N + R	IP	2.0×10^7	2.0×10^7	28	0	100
AL09-71 N + R	IP	2.0×10^6	2.0×10^7	28	0	100
AL09-71 N + R	IP	2.0×10^5	2.0×10^7	28	75	25
AL09-71 N + R	IP	2.0×10^4	2.0×10^7	28	80	20
AL09-71 N + R	IP	2.0×10^3	2.0×10^7	28	92	8
Sham TSB	IM[d]	–	2.0×10^7	28	100	–
AL09-71 N + R	IM	2.0×10^7	2.0×10^7	28	62	38
AL09-71 N + R	IM	2.0×10^6	2.0×10^7	28	88	12
AL09-71 N + R	IM	2.0×10^5	2.0×10^7	28	96	4

[a] Vaccination dose for IP and IM was in the unit of CFU/fish and CFU/mL, respectively.
[b] Days post vaccination.
[c] Intraperitoneal injection.
[d] Bath immersion.
[e] Relative percent of survival.

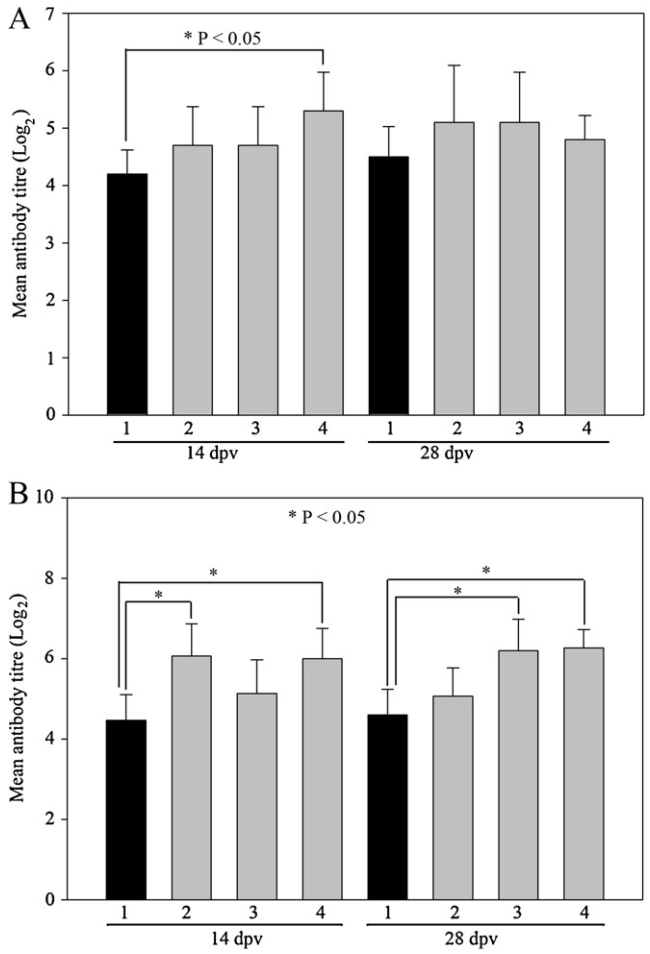

Fig. 3. Mean agglutinating antibody titers of serum samples collected from vaccinated or non-vaccinated fish. (A) Channel catfish. (B) Nile tilapia. 1, TSB sham vaccination control; 2, AL09-71 N + R vaccinated; 3, AL09-72 N + R vaccinated; 4, AL09-73 N + R vaccinated. Antibody titer was calculated as $\log_2$ of the reciprocal of the highest dilution of serum positive for agglutination. Data were presented as mean ± S.D. from fifteen serum samples.

jejuni, the leading cause of food-borne bacterial illness in the USA responsible for 31% of laboratory-confirmed gastroenteritis in 2008 [45], has a slower growth rate than that of its parent strain, with an average doubling time of 136 min vs 112 min for the parent strain [44]. Taken together, our results suggest that decreased virulence and reduced growth rate were fitness costs associated with their resistance to N + R.

At present, no vaccine is commercially available to protect farm raised fish against *A. hydrophila* infections in the US, although many studies have demonstrated that inactivated bacterins or attenuated live *A. hydrophila* are able to provide protection. This is mainly due to the fact that *A. hydrophila* is very heterogeneous both biochemically and serologically, presenting one of the biggest obstacles in developing effective commercial vaccines against *A. hydrophila* [27,32]. To control the MAS disease outbreaks in West Alabama, it is necessary to develop vaccines specifically against the strains isolated from the particular geographical location during disease outbreak. However, whether the N + R mutants offer any protection against heterologous *A. hydrophila* isolates from other geographical areas merits further study.

Studies on protection duration provided by vaccination revealed that the N + R mutants offered excellent protection to channel catfish at 14 dpv but relatively low protection at 28 and 56 dpv, suggesting that booster immunization might be necessary to offer longer and higher protection to channel catfish. However, N + R mutants offered excellent protection to Nile tilapia for as long as 56 days, suggesting that N + R mutants could be used as novel vaccines to protect Nile tilapia against *A. hydrophila* infections. By bath immersion, N + R mutants offer significantly higher protection to channel catfish than to Nile tilapia, suggesting that the rates of entry of these mutants to channel catfish and Nile tilapia might be different. Nonetheless, bath immersion of channel catfish in the N + R mutants offered significant protection to vaccinated fish against challenges by virulent parents. Further studies should be carried out to find the best formulation(s) of N + R mutants to be administered by bath immersion for both channel catfish and Nile tilapia so that they will offer up to 100% protection as provided by IP vaccination route.

Agglutination assay revealed that average antibody titers of N + R mutants-vaccinated fish (pre-challenge) at both 14 and 28 dpv were higher than those of sham vaccinated fish, suggesting that antibody-mediated immunity played a partial role in the protection elicited by the vaccination. However, the antibody titer of AL09-71 N + R mutant-vaccinated fish was not significantly different from the TSB sham vaccination control at 14 dpv, whereas the vaccination offered 100% protection to channel catfish, suggesting that other mechanisms such as cell-mediated immunity might play an important role in the protection. Cell-mediated immunity has been reported to play an important role in rainbow trout against infections [46]. However, the exact protection mechanisms elicited by the N + R-mutants in channel catfish and Nile tilapia merits further study.

In conclusion, three novel attenuated *A. hydrophila* vaccines were developed from the virulent 2009 West Alabama isolates through selection for resistance to both novobiocin and rifampicin. Vaccination of channel catfish with these mutants at dose of 4×10^5 CFU/fish offered 86–100% protection against their virulent parents at 14 dpv. Vaccination of Nile tilapia with these mutants at dose of 2×10^8 CFU/fish offered 100% protection against their virulent parents at 14, 28, and 56 dpv. Agglutination assay results suggested that protection elicited by these mutants was partially due to antibody-mediated immunity. Taken together, our results suggest that the three attenuated vaccines might be used to protect channel catfish and Nile tilapia against the highly virulent 2009 West Alabama isolates of *A. hydrophila*.

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